Cloning of Tumor-associated Differentially Expressed Gene-14, a Novel Serine Protease Overexpressed by Ovarian Carcinoma


INTRODUCTION

Serine proteases comprise a family of protein-degrading enzymes that serve as a host of biological functions, including activation of blood coagulation cascades, activation of growth and angiogenic factors, and degradation of extracellular matrix components (1–4). In recent years, aberrant expression of serine proteases such as plasminogen activator has been shown to correlate positively with the invasiveness and metastatic potential of tumor cells (3, 5, 6). Presumably, this occurs because the ability of the tumors to degrade extracellular matrix is increased, either directly or indirectly through the proteolytic activation of otherzymogenic proteases. More significantly, the serine protease known as PSA (7) has been used successfully as a tumor marker for the early diagnosis of prostate cancer due to its abnormal prevalence in the peripheral blood of these patients (7). Serine proteases play important roles in the cascade of events involved in the malignant process, and at least for prostate cancer, they provide sufficient signal to allow detection of early disease.

This year, the American Cancer Society predicts that there will be 14,500 ovarian cancer-related deaths in the United States and that there will be 25,400 new cases diagnosed (8). Unfortunately, although diagnostic assays based on the ovarian cancer antigen CA125 have improved physicians’ ability to diagnose and monitor recurrence of ovarian carcinoma, the problem remains that most of these new cases will be diagnosed at late stages, in which the primary tumor has progressed to a metastatic state. This failure to diagnose patients in the early stages of ovarian cancer directly impacts the 5-year survival rate for ovarian cancer patients, which remains below 50% (8). This study is the result of a strategy to identify those serine proteases that are overexpressed by ovarian carcinomas in an effort to define potential tumor markers.

All serine proteases contain conserved histidine, aspartate, and serine residues that are necessary for enzymatic activity. To identify the expressed serine proteases, we used degenerate oligodeoxyribonucleotide primers designed to the conserved amino acid sequences surrounding the invariant His and Ser residues of the catalytic triad (9) in PCRs with cDNA from either normal ovarian tissue or ovarian carcinoma as the template. PCR products of the appropriate size were subcloned into T-vector and sequenced. Previously, this strategy has proved successful in identifying the serine proteases hepsin and stratum corneum chymotryptic enzyme, which have been shown to be expressed at abnormally high levels in ovarian carcinoma (10). Homology searches revealed that one of the subclones obtained from ovarian carcinoma represented a novel 406-bp sequence that has significant sequence similarity to other known proteases, including mouse neuropsin, hHk2, and human PSA. The complete cDNA for this novel sequence was cloned and found to encode a trypsin-like serine protease, named TADG14. More importantly, the TADG14 transcript was found to be highly expressed in a majority of ovarian tumors but not expressed by normal ovarian tissue. High-level expression of TADG14 appears to be restricted to tumors, and this protease appears to be secreted in a manner that would suggest a possible role in invasion and metastasis. Moreover, due to the extracellular nature of this enzyme, it may be possible to exploit its expression as a diagnostic tool for ovarian cancer.

MATERIALS AND METHODS

mRNA Isolation and cDNA Synthesis. Forty-one ovarian tumors and 10 normal ovary specimens were obtained from surgery and frozen in liquid nitrogen or purchased from the Cooperative Human Tissue Network. mRNA isolation was performed according to the manufacturer’s instructions using the oligo(dT) chromatography-based Mini RiboSep Ultra mRNA isolation kit (Becton Dickinson, Bedford, MA).

First strand cDNA was synthesized using 5.0 µg of mRNA and either random hexamer or oligo(dT) primers according to the manufacturer’s protocol using a first strand synthesis kit (Clontech, Palo Alto, CA). The purity of the cDNA was evaluated by PCR using primers specific for the p53 gene. These primers span an intron such that the PCR products generated from pure cDNA can be distinguished from cDNA that is contaminated with genomic DNA.

PCRs. Reactions with degenerate primers and quantitative PCRs were carried out as described previously (10). The sequences of the TADG14-
Fig. 1. Northern blot analysis. A, mRNA was isolated from the tissues of interest as described (10) and subjected to Northern hybridization using a random-labeled 230-bp TADG14-specific RT-PCR product. The blot was stripped and probed for β-tubulin. B–D, MTN blots (Clontech) were probed with the same TADG14- and β-tubulin-specific RT-PCR products. TADG14 mRNA was detected as a 1.4-kb transcript in tumors and was not detected in any normal tissue studied.

**DNA Sequencing.** Using a plasmid-specific primer near the cloning site, we carried out sequencing reactions using Prism Ready Reaction Dye Deoxy terminators (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Residual dye terminators were removed from the completed sequencing reaction using a Centri-Sep spin column (Princeton Separation, Adelphia, NJ). An Applied Biosystems model 373A DNA Sequencing System was used for sequence analysis. Sequences were compared with the GenBank/European Molecular Biology Laboratory databases using the FASTA program (Wisconsin Package Version 9.1; Genetics Computer Group, Madison, WI). Multiple sequence alignments were generated with the Bestfit and Pileup programs, available through Genetics Computer Group.

**T-Vector Ligation and Transformations.** The purified PCR products from the PCR with degenerate primers were ligated into the Promega T-vector plasmid, and the ligation products were used to transform JM109 competent cells, according to the manufacturer’s instructions (Promega, Madison, WI). Positive colonies were cultured for amplification, the plasmid DNA was isolated by means of the Wizard Minipreps DNA purification system (Promega), and the plasmids were digested with ApsI and SacI restriction enzymes to determine the size of the insert. Plasmids with inserts of the size(s) visualized by the previously described PCR product gel electrophoresis were sequenced.

**Northern Blot Analysis.** mRNAs (~5 µg) were size-separated by electrophoresis through a 6.3% formaldehyde-1.2% agarose gel in 0.02 M MOPS, 0.05 M sodium acetate (pH 7.0), and 0.001 M EDTA. The mRNAs were then blotted to Hybond-N (Amersham, Piscataway, NJ) by capillary action in 20× SSPE. The mRNAs were fixed to the membrane by baking for 2 h at 80°C. Additional MTN blots were purchased from Clontech. These blots include the human brain MTN III blot, the human MTN II blot, the human fetal MTN II blot, and the human fetal MTN III blot. Additional MTN blots were purchased from Clontech. These blots include the human brain MTN III blot, the human MTN II blot, the human fetal MTN II blot, and the human brain MTN III blot.

The 230-bp TADG14-specific PCR product was radiolabeled using the Prime-a-Gene Labeling System, available from Promega. The blots were probed and stripped according to the ExpressHyb Hybridization Solution protocol, available from Clontech.

**Antibody Production and Western Blot Analysis.** Polyclonal antibodies were generated by immunization of white New Zealand rabbits with one of three poly-lysine-linked multiple antigen peptides derived from the deduced amino acid sequence of TADG14. These sequences are 5′-ACAG-TACGCTCTTGGAGACCA-3′ and 5′-CTGAGAGGTGAATCTTCTT-3′. The sequences of the tubulin primers that produce the 454-bp product were as follows: 5′-CGCATCAACGTGTACTACAA-3′ and 5′-ACAG-GACTTCTGGTGTACGT-3′. The critical residues of the catalytic triad are underlined, the cDNA sequence of TADG14 is shown with its deduced 260-amino acid sequence represented by the one-letter code for amino acids that are identical in at least three of the five sequences, amino acids that are similar among at least three of the five sequences.

Serine protease and ovarian cancer

**Fig. 2.** cDNA and deduced amino acid sequences of TADG14 and comparison of predicted TADG14 sequence with known proteases. A, the cDNA sequence of TADG14 is shown with its deduced 260-amino acid sequence represented by the one-letter code for each residue. Within the cDNA, the Kozak’s consensus sequence (ACCATGG) can be seen at the translation start site, whereas the underlying portion represents the polyadenylation signal. The TADG14 protein sequence contains a signal sequence near its NH2 terminus (WMFLLIIIGWAG). The critical residues of the catalytic triad are circled, whereas a potential glycosylation site (NSS) is boxed, *, the stop codon. B, using the Genetics Computer Group Pileup program, the amino acid sequence of TADG14 was compared with HMK2 (accession no. P08607), human PSA (hPSA), accession no. P07288), mouse neuropsin (mNeur), accession no. P07288), and human protease M (bProM, accession no. U62801). Black shading, amino acid residues that are identical in at least three of the five sequences. Grey shading, amino acids that are similar among at least three sequences.
buffer (pH 6.0). The specimens were incubated in methanol with 0.3% H$_2$O$_2$-stained with ethidium bromide. In this figure, the 454-bp band represents the experiment. The reaction products were electrophoresed through a 2% agarose gel and products were quantitated as described (10).

Negative controls were performed by using normal serum instead of the primary antibody. Overexpression was defined as $\pm$2SD over the mean normal values.

**RESULTS**

After confirming that the 406-bp PCR product was unique and was appropriately conserved to fit into the serine protease family, we used this PCR product as a probe for Northern blot analysis to determine the transcript size and tissue specificity of its expression. It was found that the mRNA for this clone is $\sim$1.4 kb (Fig. 1A) and that it is strongly expressed in ovarian carcinomas but not in normal ovary. More importantly, the transcript was found to be undetectable by Northern analysis in 28 normal human tissues studied (Fig. 1, B–D; data not shown). In a more sensitive assay of 50 normal human tissues (Clontech), RNA dot blot analysis revealed that this clone was very weakly expressed in only 3 of these 50 tissues: the kidney, the lung, and the mammary gland (data not shown).

Using standard hybridization techniques, we screened a cDNA library that was constructed from the mRNA isolated from the ascites cells of an ovarian cystadenocarcinoma patient. Five clones were obtained, two of which overlapped and spanned 1343 nucleotides (Fig. 2A). The last two nucleotides prior to the poly(A) tail and the poly(A) tail itself were obtained from the National Center for Biotechnology Information EST database (accession no. AA343629). Subsequent Northern blot analyses with probes derived from sequences near the 5' or 3' end of this cDNA were consistent with previous results suggesting that the obtained clones were produced by the same gene (data not shown). This cDNA includes a Kozak's consensus sequence for the initiation of translation and a polyadenylation signal. The mRNA provides an open reading frame of 260 amino acids, which contains the necessary residues (His$^73$, Asp$^{120}$, and Ser$^{212}$) in the appropriate context to classify this protein as a trypsin-like serine protease (11). Near its NH$_2$ terminus, the predicted protein contains a stretch of hydrophobic amino acids that probably serves as a secretion signal sequence (12). In addition, residues 110–112 encode a potential site for glycosylation that is common to serine proteases of the kallikrein subfamily, such as PSA. This enzyme was named TADG14, and the sequence was submitted to GenBank (accession no. AF055982).

Comparison of the deduced TADG14 amino acid sequence with sequences of known proteases revealed that it possesses significant similarity with hHK2, PSA, protease M, and mouse neuropsin (13–16). At the amino acid level, TADG14 is 48% identical to protease M,
To characterize the extent and frequency of expression of the TADG14 gene in ovarian tumors, we used semiquantitative PCR with cDNA derived from normal ovary, ovarian carcinoma, or LMP tumors as template. This technique has been previously authenticated and verified by Northern blot, Western blot, and immunohistochemistry (10, 18). PCR primers that amplify a TADG14-specific 230-bp product were synthesized and used simultaneously in reactions with primers that produce a specific 454-bp PCR product for β-tubulin. A radiolabeled nucleotide was included in this reaction, the PCR products were separated on a 2% agarose gel, and the intensity of each radiolabeled nucleotide was included in this reaction, the PCR product was synthesized and used simultaneously in reactions with primers that produce a specific 454-bp PCR product for β-tubulin. A radiolabeled nucleotide was included in this reaction, the PCR products were separated on a 2% agarose gel, and the intensity of each band was quantitated by a PhosphoImager (Molecular Dynamics). The antiserum raised to the peptide sequence LDWIKKIIG-MSK near the COOH terminal (amino acids 249–260) was used in Western blot analysis to determine whether this antibody would recognize a protein of the predicted size of M₆, 28,000. Proteins from the HeLa cell line and the carcinoma-derived MD-MB435S cell line were used in this experiment, and it was found that the antibody recognized a single M₆, 30,000 protein in both (Fig. 4, two right lanes). This size is within a reasonable range of the predicted molecular weight. As a negative control, duplicate HeLa and MD-MB435S lysates were examined with rabbit preimmune serum (Fig. 4, left two lanes). More importantly, this experiment was reproducible with antisera to a peptide from a different region of TADG14, suggesting that these cultured cancer cells produce the TADG14 protein.

Immunohistochemical staining supported the data obtained by quantitative PCR and by Northern blot. Using a TADG14 peptide-directed antibody (T14-1; described above), we observed no staining with normal ovarian tissue samples (Fig. 5A). However, intense staining was associated with tumor cells of all of the various histological subtypes of ovarian carcinoma examined. For serous carcinoma (Fig. 5, B and C), the antigen appears to be associated with tumor cells in the form of granules. These granular structures may be intermediates in the pathway that ultimately leads to secretion of TADG14. In mucinous and clear cell carcinoma samples (Fig. 5, D and F, respectively), TADG14 is highly associated with the tumor cells. In endometrioid carcinoma (Fig. 5E), the antigen is most prevalent in the glandular lumen formed by the tumor cells.

DISCUSSION

The lethality of neoplastic cells lies in their ability to proliferate abnormally and invade normal host tissues. Malignancies use proteases to provide a variety of services that assist in the process of tumor progression, including activation of growth and angiogenic factors, and to provide the basis for invasion and metastasis. In the process of studying these enzymes, we have identified overexpression of the known proteases, hepsin and stratum corneum chymotryptic enzyme. In this study, we have cloned a cDNA encoding a novel serine protease, TADG14. This protease was found to be very highly expressed in 67% (20 of 30) of ovarian carcinomas studied, whereas...
it was undetected in normal ovarian tissue. We were also unable to detect the TADG14 transcript in any of 50 normal human tissues studied. Upon prolonged Northern blot exposure, extremely low levels of TADG14 were detected in normal kidney, breast, and lung. This suggests the possibility that this gene is under the control of a promoter that is most active in ovarian tumors, and it may be possible to exploit this for therapeutic means. Unfortunately, TADG14 expression can be detected in other types of cancer, including prostate, breast, and colon. This may limit the usefulness of TADG14 as a potential diagnostic marker for ovarian carcinoma, but it in no way detracts from the usefulness of this molecule as a target for cancer therapy or the usefulness of the TADG14 promoter in gene therapy applications.

At the nucleotide level, TADG14 mRNA resembles the recently cloned human neuropsin transcript, with obvious differences residing in the 5' and 3' UTRs. TADG14 mRNA contains 491 bases of 5' UTR that were not found in human neuropsin. Also, the nucleotides preceding the poly(A) tail in the 3' UTR are not homologous. A 0.9-kb transcript for human neuropsin was identified in cultured keratinocytes but not in normal hippocampus. Also, it was not identified as being associated with tumors. At the amino acid level, TADG14 is identical to human neuropsin. Among other known proteases, TADG14 most closely resembles the mouse protease known as neuropsin, which was originally cloned from mouse hippocampus and, subsequently, implicated in neuronal plasticity (19). If TADG14 functions in a manner similar to mouse neuropsin, it may be capable of restructuring the three-dimensional architecture of a tumor, allowing for shedding of tumor cells or invasion of normal host tissues by degrading fibronectin (20). In support of this, immunohistochemical staining of ovarian tumors revealed that TADG14 is highly associated with tumor cells and the cells near the invasive fronts of tumor. Therefore, TADG14 could be an important target for the inhibition of tumor progression. Most importantly, the 5-year survival rate for ovarian cancer patients remains <50% because of an inability to diagnose this disease at an early stage. TADG14 contains a secretion signal sequence and immunohistochemical data suggest that TADG14 is secreted. In addition, by Northern blot and RNA dot blot analyses, TADG14 appears only in abundance in tumor tissues. As a result of this, it may be possible to design assays based on the detection of this protein for the early detection of ovarian cancer. Currently, the best available ovarian cancer tumor marker is CA125. However, due to high endogenous circulating levels of this antigen, the signal:noise ratio limits its usefulness as a diagnostic tool. Therefore, TADG14, due to its limited expression in other tissues and potential for being present in the circulation of tumor-bearing patients, may prove to be a useful tool for early detection of ovarian cancer, especially the most prevalent serous cystadenocarcinoma subtype.

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